JOURNAL OF BACTERIOLOGY, Mar. 1982, p. 1096–1101
0021-9193/82/031096-06$02.00/0

Vol. 149, No. 3

Naphthalene Plasmids in Pseudomonads

MARGARET A. CONNORS AND E. A. BARNSLEY*

Department of Biochemistry, Memorial University of Newfoundland, St. John's, Newfoundland, Canada A1B 3X9

Received 15 May 1981/Accepted 21 October 1981

A rapid method beginning with the direct lysis of bacteria in alkaline sodium dodecyl sulfate was used to detect naphthalene plasmids in pseudomonads. The strains NCIB 9816, P_G, ATCC 17483, and ATCC 17484, which can grow on naphthalene as the sole source of carbon and energy, were examined. All except ATCC 17483 contained more than one plasmid. ATCC 17483 did not contain any plasmids. The largest pair of plasmids found in each of NCIB 9816 and P_G (NAH2 and NAH3, respectively) determined naphthalene metabolism and could be transferred by conjugation. This also transferred the unusually regulated *meta* pathway enzymes for catechol metabolism. NAH2 determines the constitutive production of low concentrations of catechol 2,3-dioxygenase and 2-hydroxymuconic acid semialdehyde dehydrogenase, and NAH3 determines the constitutive production of high concentration of these. NAH2 and NAH3 gave identical fragments on digestion with *Bam*HI or *Hin*dIII, but these were quite different from those of NAH. Nonetheless, NAH2 and NAH3 hybridized with NAH.

Three plasmids which determine the degradation of naphthalene in pseudomonads have been described. NAH (10) specifies the early enzymes of naphthalene metabolism and probably those for later steps involving the degradation of catechol by the *meta* pathway. NPL1 (6), on the other hand, allows growth on naphthalene only when a separate specification for salicylate metabolism is present. pND 140 and pND 160 (9) have been distinguished from NAH by their insensitivity to induction by 3-methylcatechol. NAH has been isolated, and its molecular weight has been determined (14); NAH has been compared with other catabolic plasmids (4, 11, 13). Plasmids have been detected in three other pseudomonads able to degrade naphthalene, ATCC 17484, NCIB 9816, and P_G, and the loss of a plasmid from ATCC 17484 and from NCIB 9816 was associated with the loss of the ability to degrade naphthalene (J. B. Johnston, personal communication).

We have now examined the genetic basis for naphthalene metabolism in these strains which we earlier characterized biochemically (3). The transfer of naphthalene metabolism by conjugation was possible with P_G and NCIB 9816, but not with ATCC 17483 and ATCC 17484. (We were also not able to cure the latter two organisms.) Plasmids were detected in lysates of PpG7, P_G, NCIB 9816, and ATCC 17484, but several methods (5, 12, 15, 17) gave poorly reproducible results. Other laboratories have reported difficulty in isolating catabolic plasmids (17, 21). This problem was avoided in two ways. Where possible, plasmids were transferred to *Pseudomonas putida* PaW330, from which isolation is reproducible by any method (C. J. Duggleby, personal communication). The method of Birnboim and Doly (5) was also modified slightly to give reproducible isolation of catabolic plasmids. It was then possible to show that NCIB 9816, P_G, and ATCC 17484 each contains several plasmids. Naphthalene degradation in NCIB 9816 and P_G is determined by the largest plasmid in each organism which we have designated NAH2 and NAH3, respectively. On electrophoresis, NAH, NAH2, and NAH3 are not separated. NAH2 and NAH3 give distinctly different patterns from NAH on analysis with restriction endonucleases, but hybridize extensively with NAH. We were not able to confirm the report (13) of a plasmid in ATCC 17483.

MATERIALS AND METHODS

Bacterial strains. We obtained *P. putida* PpG7 from I. C. Gunsalus, *Pseudomonas* sp. strain P_G from P. A. Williams, PaW330 from C. J. Duggleby, and AC545 from A. M. Chakrabarty. NCIB 9816 was obtained from the National Collection of Industrial Bacteria, Torrey Research Station, Aberdeen, Scotland.

Growth on naphthalene was tested by inoculating petri dishes containing agar-mineral salts (19) and, where necessary, a required amino acid (100 μg/ml) and by incubating them at 25°C with naphthalene in the lid. To obtain liquid cultures grown on naphthalene, we found it more convenient to use the following procedure rather than one described previously (3). Sterile molten naphthalene (about 1 ml) was poured into 250 ml of a sterile salt solution (16) containing 0.7

mM succinate and any required amino acid (100 μg/ml). The medium was shaken during the addition to disperse the naphthalene. The succinate present allowed limited growth (absorbance at 600 nm between 0.15 and 0.2), and such cultures grown overnight at 25°C usually had an absorbance between 0.4 and 0.8 and were still growing. These cultures are referred to as being grown on naphthalene, even though the growth was initiated on succinate. Cultures grown entirely on succinate contained 7 mM succinate and the required amino acid (100 μg/ml) in the same mineral salts.

Measurement of enzymic activities. Cultures were harvested and washed, and cells were disrupted by sonication as described previously (2). Catechol 2,3-dioxygenase and catechol 1,2-dioxygenase (3) were measured as described previously, except that the sonicated cells were not centrifuged. Catechol 1,2-dioxygenase was measured after inactivation of the 2,3-dioxygenase with the minimum of H_2O_2 (3).

Auxotrophic mutants. A mid-log-phase culture growing on succinate was centrifuged, suspended in mineral salts to give an absorbance at 600 nm of 0.2, and incubated with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (20 μg/ml) at 25°C for 30 min. Between 4 and 40% of the cells remained viable on nutrient agar at this stage. The suspension was centrifuged, resuspended in mineral salts plus succinate and amino acid (100 μg/ml), and grown overnight. The culture was then taken through three cycles of the procedure of Ornston et al. (16) to enrich the culture with respect to amino acid auxotrophs. They were finally selected by replica plating.

Conjugation. The donor was grown to the stationary phase at 25°C in nutrient broth and diluted 10-fold into fresh medium. After a 0.5-h incubation, 0.5 ml of donor culture and 0.5 ml of a stationary-phase culture of the recipient were mixed in a tube and incubated (without shaking) at 25°C for 30 to 45 min. The culture was then centrifuged, washed with 1% NaCl, diluted, and spread on selective media to count donors and exconjugates separately.

Extraction and purification of plasmid DNA. Plasmid DNA was extracted from exconjugates of PaW330 by the method of Meyers et al. (15) from volumes of nutrient broth-culture up to 1 liter, which were harvested when the absorbance at 600 nm reached 2. The volumes of reagents used were proportional to those originally described (15). For deproteinization with phenol, the material from 1 liter of culture was divided between eight 30-ml Corex centrifuge tubes. After deproteinization, the aqueous layers were recombined in a 500-ml sterile flask for precipitation of the DNA with ethanol. After the preparation was allowed to stand at −20°C, DNA was removed by centrifugation at −10°C in two 30-ml Corex tubes. Each supernatant was poured off, the tubes were topped up and centrifuged, and the final pellet obtained in each tube was dissolved in 1 ml of TES (15). Preparations were frozen at −20°C until used.

Plasmid DNA was purified by centrifugation in CsCl-ethidium bromide solution. The nucleic acid content of the crude preparation was measured by diluting 10 μl of the preparation into 3.0 ml of phosphate buffer (pH 7) and by measuring the increase in absorbance at 260 nm. The absorbance of the undiluted preparation was calculated (usually about 360), and a volume was then used for purification so that the product of the volume and the calculated absorbance was 144. The following steps were carried out under a dull red light, and the material was not exposed to white light until after the final dialysis. We mixed for centrifugation 6.0 ml of CsCl (saturated aqueous solution), 1.0 ml of ethidium bromide (3 mg/ml), and the sample diluted to 2.0 ml with TES. The sample of the crude preparation was then diluted to 2.0 ml with *N*-tris (hydroxymethyl) methyl-2-aminoethanesulfonic acid. The refractive index was adjusted to 1.3900 ± 0.0005 at 21°C, and the solution was centrifuged at 40,000 rpm for 40 to 43 h at 15°C (Beckman L3-50 centrifuge, 50 Ti head).

The gradient was collected in fractions of 0.2 ml each, and a 20-μl sample of each of these was electrophoresed in a 0.5% agarose gel (type V agarose, Sigma Chemical Co., St. Louis, Mo.) in Tris-borate buffer (15) at a field strength of 5 V/cm until a marker of bromophenol blue had run 10 cm.

The gel was stained with ethidium bromide in water (0.4 μl/ml), viewed over a C-61 transilluminator (Ultra-Violet Products, Inc., San Gabriel, Calif.), and photographed on Kodak high-contrast copy film 5069 through a Hoya 0 (g) filter.

Those fractions from the gradient which contained only plasmid DNA were combined, and ethidium bromide was extracted with propan-2-ol saturated with water and NaCl. The preparation was then dialyzed against three separate lots of 600 ml of 1 mM Tris–0.1 mM EDTA buffer (pH 8.0) at 5°C in bottles wrapped in aluminum foil to exclude light. Only after the final dialysis was the preparation exposed to white light. It was transferred to a tube, the dialysis sack was rinsed with a little water, and the volume was then reduced approximately 10-fold by extraction with butan-2-ol. The preparations were divided into small portions before freezing at −20°C.

Detection of plasmids in wild-type strains. A 1.5-ml sample of a culture growing exponentially in nutrient broth (absorbance 1.0 to 1.2) was centrifuged for 2 min at room temperature in an Eppendorf model 5412 centrifuge. The pellet was resuspended in 1 ml of ice-cold 25% (wt/vol) sucrose–10 mM Tris (pH 7.2), and centrifuged again. The pellet was resuspended in 0.1 ml of sucrose-Tris and lysed with 0.3 ml of a solution containing 1% sodium dodecyl sulfate–0.2 M NaOH–4.4 mM EDTA. After the solution had stood at room temperature for 15 min, 0.17 ml of 4 M sodium acetate–acetic acid (pH 4.8 when diluted 10-fold) was added, and the mixture was left on ice for 1 h. The suspension was centrifuged (10 min at room temperature), and the supernatant was mixed with an equal volume of isopropanol. The mixture was cooled at −18°C for at least 10 min and then centrifuged at room temperature for 5 min. The pellet was dissolved in 25 μl of buffer for electrophoresis.

Analysis with restriction endonucleases and by hybridization. Digestion with restriction endonucleases was carried out at 37°C in 20 mM Tris-hydrochloride (pH 7.0)–100 mM NaCl–7 mM $MgCl_2$ for *Bam*HI and in 20 mM Tris-hydrochloride (pH 7.4)–60 mM NaCl–7 mM $MgCl_2$ for *Hin*dIII. The digestion products were mixed with agarose beads containing bromophenol blue and were electrophoresed in a 0.8% agarose gel in Tris-borate buffer (15). Plasmids were labeled with [^{3}H]TTP (New England Nuclear Corp., nick translation system, Boston, Mass.) and hybridization carried

TABLE 1. Bacterial strains used in the transfer of naphthalene plasmids to *P. putida* PaW330

Strain no.	Origin	Phenotype	Frequency of conjugation[a]	Plasmid carried
PaW330	C. J. Duggleby	Trp^-		None
AC545	A. M. Chakrabarty	Sal^+ Met^-		SAL
MC213	Cured strain of AC545	Met^-		None
PpG7	I. C. Gunsalus	Nah^+[b]		NAH
MC86	Auxotroph of PpG7	Nah^+ Leu^-		NAH
MC281	MC86 × PaW330[c]	Nah^+ Trp	5×10^{-4}	NAH
NCIB 9816	NCIB	Nah^+		NAH2
MC190	Auxotroph of NCIB 9816	Nah^+ Leu^-		NAH2
MC256	MC190 × MC213	Nah^+ Met^-	0.028	NAH2
MC274	MC256 × PaW330	Nah^+ Trp	0.1	NAH2
P_G	P. A. Williams	Nah^+		NAH3
MC192	Auxotroph of P_G	Nah^+ Leu^-		NAH3
MC262	MC192 × MC213[c]	Nah^+ Met	0.1	NAH3
MC268	MC262 × PaW330[c]	Nah^+ Trp^-	0.04	NAH3

[a] Exconjugates as a percentage of donors.
[b] Grows on mineral salts agar over naphthalene.
[c] Exconjugates selected by providing naphthalene and the amino acid required by the recipient.

out as described by Heinaru et al. (13). The washed cellulose nitrate sheets loaded with hybridized DNA were dried and dipped in toluene containing PPO (2,5-diphenyloxazole; 20%), and an autofluorograph was prepared with Curix RP1 X-ray film (Agfa-Gavaert, Toronto, Ontario, Canada).

RESULTS AND DISCUSSION

The strains used in this work are listed in Table 1. MC86, a leucine auxotroph derived from PpG7, was the only organism we could cross directly with PaW330. MC190 and MC192, leucine auxotrophs of NCIB 9816 and P_G, respectively, did not conjugate at a detectable frequency (<1 in 10^8) either in liquid medium or on nutrient plates. These strains conjugated readily with MC213, however, and the progeny of these crosses conjugated with PaW330. The plasmids present in the original wild-type strains were detected by our modification of the method of Birnboim and Doly (5). The modification essentially eliminates the initial incubation with lysozyme, and it is applicable to many gram-negative organisms, although occasionally certain uncharacterized isolates from soil have failed to give clear lysates on treatment with alkaline sodium dodecyl sulfate.

P_G, NCIB 9816, and ATCC 17484 each contains more than one plasmid (Fig. 1). The largest of these in each strain is similar in size to NAH found in PpG7, and it is this plasmid which is transferred from P_G and NCIB 9816 on conjugation and selection for Nah^+. We presume the largest plasmid in ATCC 17484 may determine naphthalene metabolism by that organism, but we have not been able to demonstrate its role by either conjugation or curing. With ATCC 17483, we have not been able to confirm the previous report (13) of the presence of a plasmid identical to NAH. ATCC 17483 is readily characterized biochemically. It does not grow on benzoate and during growth on naphthalene does not produce any of the enzymes for the *ortho* pathway of catechol metabolism (3). Although our method detects catabolic plasmids in other strains very reproducibly, we have not been able to detect one in ATCC 17483. We have not been able to cure this organism (although attempts to do so with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine leads to mutations in naphthalene metabolism with high frequency [7]), and we have not been able to transfer the Nah^+ character by conjugation.

The early enzymes of naphthalene metabolism in P_G are induced during growth on naphthalene or salicylate and induced gratuitously by 2-aminobenzoate during growth on succinate (3), and a similar behavior is observed with NCIB 9816 (2). The enzymes for the oxidation of catechol by the *meta* pathway behave quite differently in these organisms. Those of P_G are constitutive with high levels of activity, whereas those of NCIB 9816 have a low activity under all conditions of growth (3). Because these organisms are putatively identical it was suggested there might be two operons for the regulation of naphthalene metabolism, one for early enzymes and one for those of catechol metabolism through the *meta* pathway. Austen and Dunn (1) have produced experimentally in a plasmid regulatory mutants which demonstrate this possibility. Our present observations on the naphthalene plasmids from NCIB 9816 and P_G (NAH2 and NAH3, respectively) show that transfer of the plasmids to new hosts is accompanied by the transfer of characteristic biochemical properties (Table 2). Thus, NAH2 in MC190 and MC274

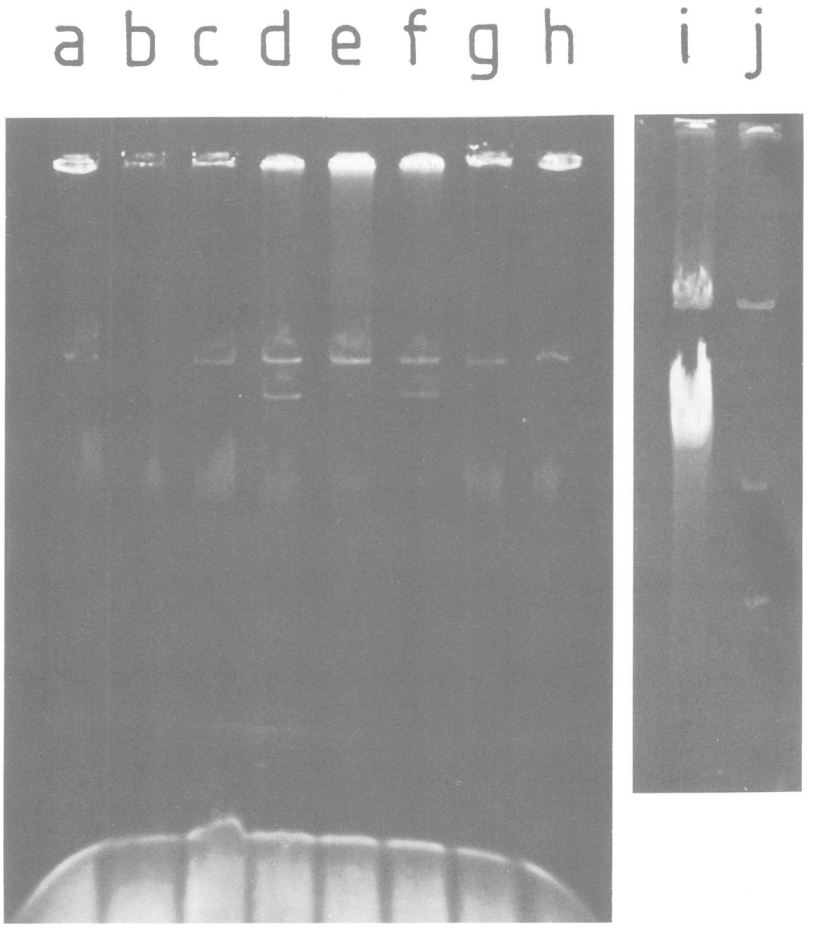

FIG. 1. Naphthalene plasmids in their original hosts and after transfer to PaW330. The tracks contain: a, PpG7; b, PaW330; c, MC268; d, P_G; e, MC274; f, NCIB 9816; g, MC281; h, PpG7; i, MC281; and j, ATCC 17484.

TABLE 2. Activities of *meta* pathway enzymes in strains carrying the naphthalene plasmids NAH2 and NAH3

Strain no.	Plasmid	Carbon source	Sp act (μmol·min^{-1}·mg of protein^{-1})	
			Catechol 2,3-dioxygenase	2-Hydroxymuconic acid semialdehyde dehydrogenase
PaW330	None	Succinate	<0.001	<0.001
MC213	None	Succinate	<0.001	<0.001
MC190	NAH2	Succinate	0.038	0.005
		Naphthalene	0.011	0.007
MC274	NAH2	Succinate	0.005	0.006
		Naphthalene	0.003	0.005
MC192	NAH3	Succinate	0.418	0.130
		Naphthalene	0.405	0.136
MC268	NAH3	Succinate	0.410	0.125
		Naphthalene	0.425	0.131

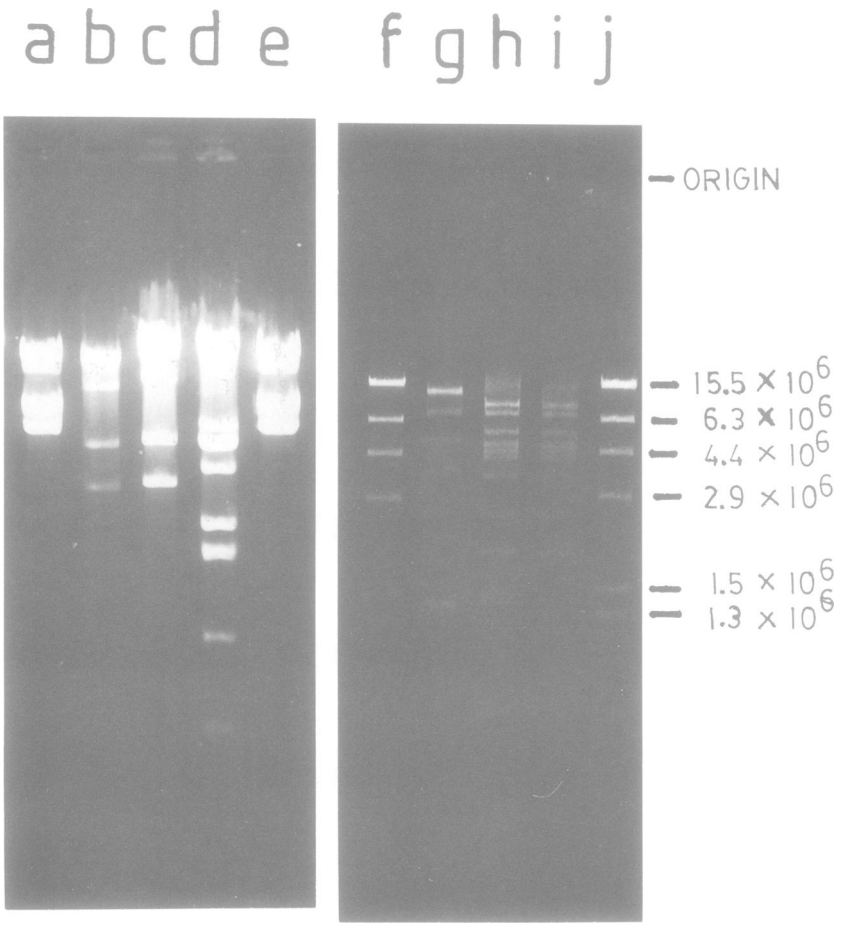

FIG. 2. Restriction patterns of naphthalene plasmids NAH, NAH2, and NAH3. *Bam*HI digests: a, λ; b, NAH2; c, NAH3; d, NAH; e, λ. *Hin*dIII digests: f, λ; g, NAH; h, NAH2; i, NAH3; and j, λ. The molecular weights given are for the *Hin*dIII digests of λ. The fragments of NAH produced by *Hin*dIII (track g) are in accord with those reported previously (11). Assuming the first intense band is two unresolved fragments as reported in reference 11, the molecular weight of fragments of NAH sum to 52×10^6. Farrell et al. reported 50×10^6 (11). The *Hin*dIII fragments of NAH2 and NAH3 sum to a molecular weight of 77×10^6.

causes the production of low activities of catechol 2,3-dioxygenase and 2-hydroxymuconic acid semialdehyde dehydrogenase which are not present in the plasmid-free strains (MC213 and PaW330, respectively), which are similar to those in the parent strain NCIB 9816 (3), and which are not induced during growth on naphthalene. NAH3 in MC192 and MC268 causes the production of high constitutive levels of the *meta* pathway enzymes, similar to those in P_G (3), but which are not present in the plasmid-free strains. We have also observed (unreported) that in all Nah$^+$ exconjugates, naphthalene oxygenase (19), 1,2-dihydroxynaphthalene oxygenase (18), salicylaldyle dehydrogenase (20), and salicylate hydroxylase (2) are induced during growth on naphthalene or salicylate in a way similar to the original wild types P_G and NCIB 9816 (2, 3). On digestion with *Bam*HI and *Hin*dIII, NAH2 and NAH3 give identical fragments (Fig. 2). This is in accord with the view that they have a common origin and suggests that laboratory propagation has fortuitously selected two different regulatory mutants.

The pattern of fragments given by NAH on hydrolysis with restriction endonucleases was markedly different from that given by NAH2 and NAH3 (Fig. 2). Nonetheless all the fragments of NAH2 and NAH3 hybridize with NAH, and all fragments of the latter hybridize with NAH2 and NAH3 (Fig. 3). It appears, therefore, that notwithstanding the different restriction patterns NAH, NAH2, and NAH3 do have structural relationships. It remains to be determined

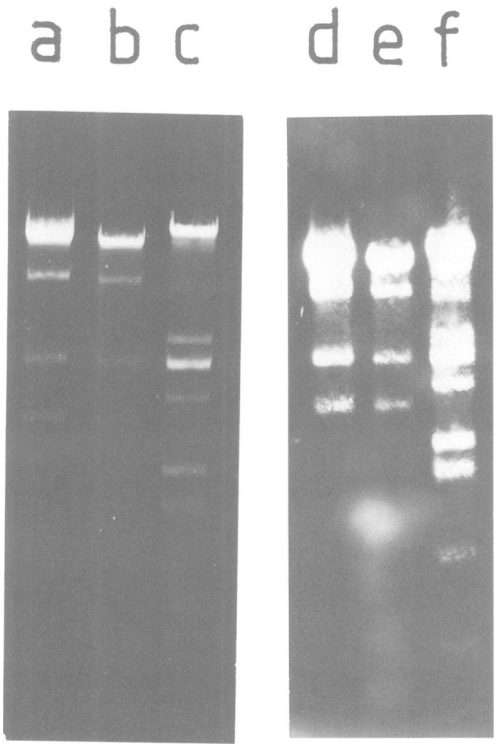

FIG. 3. Hybridization of *Bam*HI restriction fragment of NAH, NAH2, and NAH3 with NAH labeled with [^{3}H]thymine. Tracks a, b, and c, NAH3, NAH2, and NAH, respectively, in the original gel. Tracks d, e, and f, the autofluorograph of NAH3, NAH2, and NAH, respectively, after transfer by the Southern blot method.

whether these similarities lie in the structures of genes for catabolic enzymes or in the structure of other parts of the plasmids, or in both of these areas.

The molecular weight of NAH determined by the summation of the masses of fragments produced by *Hin*dIII was 52×10^6, in agreement with that reported previously (11) using this method. The molecular weight of NAH2 and NAH3 determined in this way was 77×10^6. The intact plasmids NAH, NAH2, and NAH3 are not separated on electrophoresis in 0.5% agarose (Fig. 1), and this suggests NAH2 and NAH3 are more highly supercoiled than NAH.

ACKNOWLEDGMENTS

The authors thank J. B. Johnston and C. J. Duggleby for information and advice.

LITERATURE CITED

1. **Austen, R. A., and N. W. Dunn.** 1980. Regulation of the plasmid-specified naphthalene catabolic pathway of *Pseudomonas putida*. J. Gen. Microbiol. **117**:321–328.
2. **Barnsley, E. A.** 1975. The induction of the enzymes of the naphthalene metabolism in *Pseudomonas* by salicylate and 2-aminobenzoate. J. Gen. Microbiol. **88**:193–196.
3. **Barnsley, E. A.** 1976. Role and regulation of the *ortho* and *meta* pathways of catechol metabolism in pseudomonads metabolizing naphthalene and salicylate. J. Bacteriol. **125**:404–408.
4. **Bayley, S. A., D. W. Morris, and P. Broda.** 1979. The relationship of degradative and resistance plasmids of *Pseudomonas* belonging to the same incompatibility group. Nature (London) **280**:338–339.
5. **Birnboim, H. C., and J. Doly.** 1979. A useful alkaline extraction procedure for screening recombinant plasmid DNA. Nucleic Acids Res. **7**:1513–1523.
6. **Boronin, A. M., I. I. Starovoitov, A. N. Borisoglevskaya, and G. K. Skryabin.** 1976. Plasmids of *Pseudomonas putida* which control the initial stages of naphthalene oxidation. Dokl. Akad. Nauk. SSSR **228**:962–965.
7. **Connors, M. A., and E. A. Barnsley.** 1980. Metabolism of naphthalene by pseudomonads: salicylaldehyde as the first possible inducer in the metabolic pathway. J. Bacteriol. **141**:1052–1054.
8. **Duggleby, C. J., S. A. Bayley, M. J. Worsey, P. A. Williams, and P. Broda.** 1977. Molecular sizes and relationships of TOL plasmids in *Pseudomonas*. J. Bacteriol. **130**:1274–1280.
9. **Dunn, N. W., H. M. Dunn, and R. A. Austen.** 1980. Evidence for the existence of two catabolic plasmids coding for the degradation of naphthalene. J. Gen. Microbiol. **117**:329–333.
10. **Dunn, N. W., and I. C. Gunsalus.** 1973. Transmissible plasmid coding early enzymes of naphthalene oxidation in *Pseudomonas putida*. J. Bacteriol **114**:974–979.
11. **Farrell, R., I. C. Gunsalus, I. P. Crawford, J. B. Johnston, and J. Ito.** 1978. Restriction endonuclease sites and aromatic metabolic plasmid structure. Biochem. Biophys. Res. Commun. **82**:411–416.
12. **Hansen, J. B, and R. H. Olsen.** 1978. Isolation of large bacterial plasmids and characterization of the P2 incompatibility group plasmids pMG1 and pMG5. J. Bacteriol. **135**:227–238.
13. **Heinaru, A. L., C. J. Duggleby, and P. Broda.** 1978. Molecular relationships of degradative plasmids determined by *in situ* hybridization of their endonuclease-generated fragments. Mol. Gen. Genet. **160**:347–351.
14. **Johnston, J. B., and I. C. Gunsalus.** 1977. Isolation of metabolic plasmid DNA from *Pseudomonas putida*. Biochem. Biophys. Res. Commun. **75**:13–19.
15. **Meyers, J. A., D. Sanchez, L. P. Elwell, and S. Falkow.** 1976. Simple agarose gel electrophoretic method for the identification and characterization of plasmid deoxyribonucleic acid. J. Bacteriol. **127**:1529–1537.
16. **Ornston, L. N., M. K. Ornston, and G. Chou.** 1969. Isolation of spontaneous mutant strains of *Pseudomonas putida*. Biochem. Biophys. Res. Commun. **36**:179–184.
17. **Palchaudhuri, S., and A. Chakrabarty.** 1976. Isolation of plasmid deoxyribonucleic acid from *Pseudomonas putida*. J. Bacteriol. **126**:410–416.
18. **Patel, T. R., and E. A. Barnsley.** 1980. Naphthalene metabolism by pseudomonads: purification and properties of 1,2-dihydroxynaphthalene oxygenase. J. Bacteriol. **143**:668–673.
19. **Shamsuzzaman, K. M., and E. A. Barnsley.** 1974. The regulation of naphthalene oxygenase in pseudomonads. J. Gen. Microbiol. **83**:165–170.
20. **Shamsuzzaman, K. M., and E. A. Barnsley.** 1974. The regulation of naphthalene metabolism in pseudomonads. Biochem. Biophys. Res. Commun. **60**:582–689.
21. **Williams, P. A., and K. Murray.** 1974. Metabolism of benzoate and methylbenzoates by *Pseudomonas putida* (*arvilla*) mt-2: evidence for the existence of a TOL plasmid. J. Bacteriol. **120**:416–423.